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The Secret of Viral Reproduction

George W. Cochran

Utah State University

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**THE SECRET OF VIRAL
REPRODUCTION**

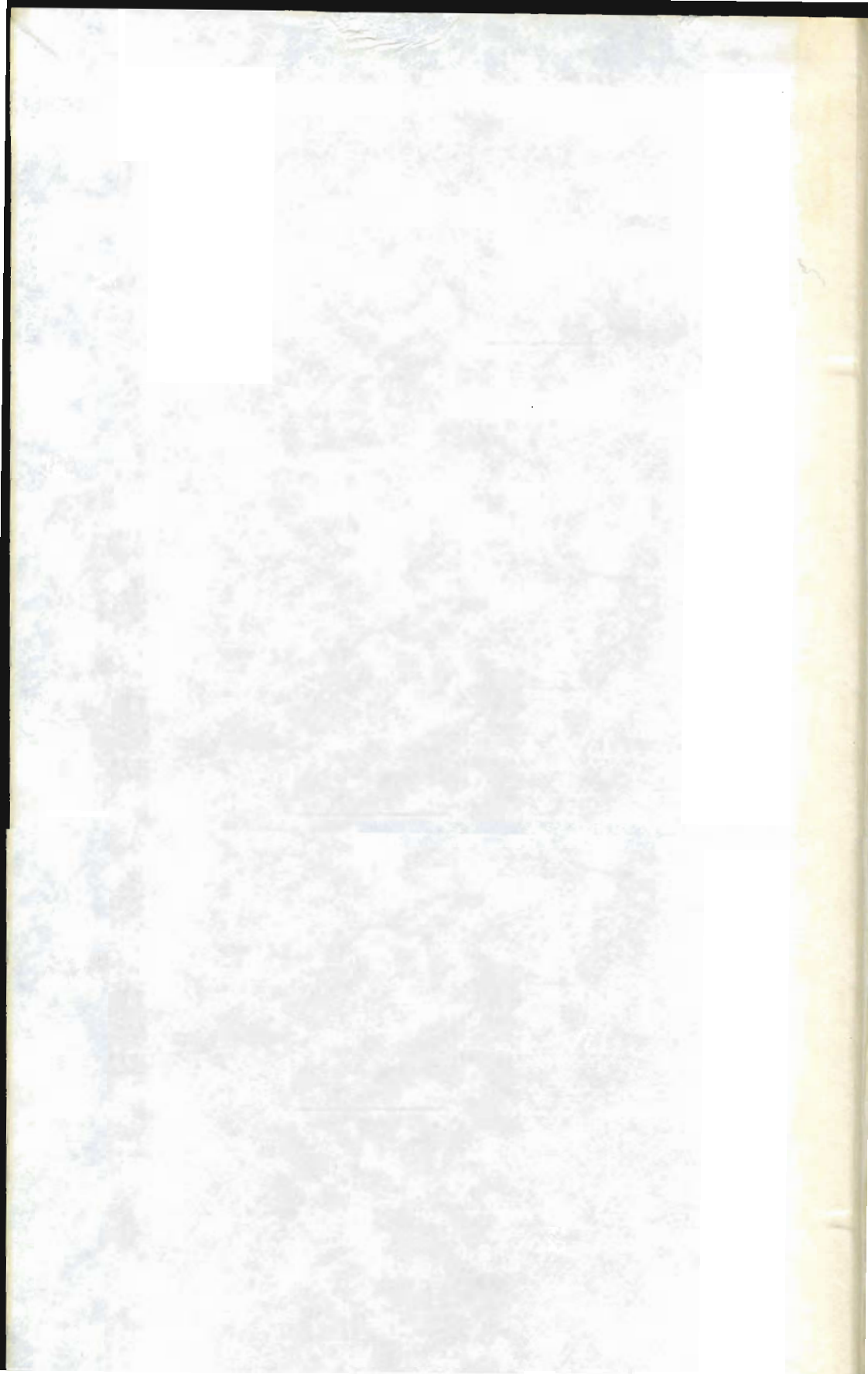
GEORGE W. COCHRAN

FACULTY HONOR LECTURE

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SECRETS OF VIRAL REPRODUCTION

A blue-toned electron micrograph showing numerous rod-shaped viral particles, likely tobacco etch virus, scattered across the field of view. The particles are uniform in size and shape, appearing as bright, slightly curved lines against a darker blue background.

COVER: Tobacco mosaic virus rods synthesized by a cell-free system containing normal bean nuclei and chloroplasts after being primed by phenol-prepared viral RNA in the presence of 19 amino acids and four ribonucleoside-5'-triphosphates.

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TWENTY-EIGHTH FACULTY HONOR LECTURE

THE SECRETS OF VIRAL REPRODUCTION

by

GEORGE W. COCHRAN

Professor of Botany and Plant Pathology

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ACKNOWLEDGEMENTS

I WISH to express my appreciation to my associates who have contributed valuable suggestions and many hours of assistance in this research effort. These include: Amrik Singh Dhaliwal, John Chidester, George W. Welkie, J. Storz, John R. Simmons, Jon H. Venekamp, Antonina Mikulska-Macheta, Hendrika Kijne-Knop, B. K. Chandrasekhar, Men Hui Lee, Helen Lu Sheng Wang, and Calvin Lamborn. This research has been supported by the Utah Agricultural Experiment Station, the United States Atomic Energy Commission, and the National Institutes of Health.

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THE SECRETS OF VIRAL REPRODUCTION

By GEORGE W. COCHRAN

THE THRESHOLD OF LIFE

NUCLEIC acid is the basis of life. There are 2 types, ribose nucleic acid (RNA) and deoxyribose nucleic acid (DNA). DNA, the material found in the chromosomes within the nuclei of the cells of most living organisms, bears the genetic code that determines all of the characteristics of a living organism. Information from the DNA of the chromosomes is carried to all parts of the cell by one type of RNA called messenger RNA. Additional specialized RNA molecules are found inside the ribosomes, tiny bodies within the protoplasm that make proteins for the cell. Still other, smaller RNA molecules, called soluble RNAs play an active role in the synthesis of proteins by linking with the building blocks (amino acids) that form new protein. The soluble RNAs participate with specialized proteins (enzymes) and the ribosomes in following the instructions of the messenger RNA to build new protein molecules.

It is not my purpose to go into further detail regarding the involvement of DNA and RNA in protein synthesis at this time. Watson (1963) in his Nobel lecture has already done this. This brief review of the functions of DNA, RNA, and protein in living cells is necessary to furnish a basis for understanding the viral research that I shall discuss in this lecture. Before any cell can divide new copies of its DNA and RNA must be made by a chemical process. In 1956, Ochoa reported the isolation of an enzyme from bacteria that was capable of synthesizing new RNA in a cell-free system in a test tube from ribonucleoside diphosphates. In 1957 Kornberg described the isolation of an enzyme from bacteria that synthesized new DNA in a cell-free system in a test tube from deoxyribonucleoside triphosphates. Ochoa and Kornberg were awarded the Nobel Prize in medicine for these discoveries in 1959. The nucleic acids formed by the Ochoa and Kornberg within the protoplasm that make proteins for the cell. Still other, smaller RNA molecules, called soluble RNAs play an active role in the synthesis of proteins by linking with the building blocks (amino acids) that form new protein. The soluble RNAs participate with specialized proteins (enzymes) and the ribosomes in following the instructions of the messenger RNA to build new protein molecules.

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At the present time most virologists believe that viruses should be classed with living organisms because they are highly complex structures with organization that can hardly be attributed to nonliving entities. They usually contain proteins and nucleic acids, the two substances that are characteristic of all living organisms. The nucleic acid is the infectious, hereditary part of the virus which contains all of the genetic information that the virus needs to carry on its cell-directing activities including its ability to produce new copies of itself. The science of genetics can be applied to viruses just as to all other living organisms, and viral genes can be located on the viral nucleic acid strands just as other genes can be located on the chromosome of higher living organisms.

The science of virology has been retarded by the small and elusive nature of the viral agents. Virologists have had to work with unseen entities which were recognized only by their ability to produce disease in other organisms. In spite of these tremendous difficulties, some remarkable advances have been made.

INFECTIVITY OF VIRAL NUCLEIC ACIDS

IN 1952 Hershey and Chase showed that the bacteriophage, virus that attacks bacteria, consists of two parts. The inner part is a nucleic acid which can be labeled with radioactive phosphorus, while the outer part is a protein which can be labeled with radioactive sulfur. Furthermore they showed that when the bacterial virus attacked a bacterial cell, the protein coat remained outside while the nucleic acid entered the bacterium to cause the viral infection. This was conclusive proof that the infective part of this virus was the deoxyribose nucleic acid (DNA). This is the type of nucleic acid that is found in chromosomes. Ribose nucleic acid (RNA) is the type that seems to be present in most plant viruses and many animal viruses. Viruses contain either RNA or DNA but not both of them within the same virus.

In 1956 Gierer and Schramm and Fraenkel-Conrat and his associates showed that the DNA of the tobacco mosaic virus is the infective part of the virus. In spite of these tremendous difficulties, some remarkable advances have been made.

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cellulose adsorbants. We obtained two types of infective virus. The first type to pass through the adsorbant was highly unstable and quickly lost its infectivity. The second type, which came off much later, was the highly stable, mature form of the virus. We postulated that the first-appearing unstable infective form was a naturally occurring viral nucleic acid and that it was probably an early stage in the virus formation process.

During the next 3 years we demonstrated the presence of this unstable viral form by two other analytical methods, agar gel electrophoresis and agar gel filtration techniques, while giving continued thought to the function that this form of the virus might play in the infection process. During this period we developed new techniques for the analysis of the virus infection by infrared spectrophotometry. In these techniques, infected and normal tissues were mounted directly in the beams of the instrument for analysis. These studies revealed remarkable biochemical changes associated with the viral infection which occurred within minutes after viral inoculation. We found that these changes apparently were associated with the viral protein coat rather than with the viral nucleic acid. This seemed to indicate that the viral protein might have some role in the infection process, but further studies will be needed to establish this. During this same period, research at the virus laboratories at the University of California and at the Max Planck Institutes for Biological and Viral Research, Tübingen, Germany, had determined the amino acid sequence of the tobacco mosaic virus protein. The nucleic acid core of one tobacco mosaic virus particle is coated by 2,200 apparently identical protein subunits. Each subunit is composed of 158 amino acids linked together in a long folded chain. While this work revealed the nature of the viral protein, the exact nature of the viral nucleic acid still remained as one of nature's secrets.

IMPROVEMENT IN PLANT VIRAL ASSAY PROCEDURES

THE reliable interpretation of plant viral research depends on the scientist's ability to assay for infective virus and viral nucleic acid in a simple and reproducible manner. In our laboratory, we were not satisfied with the results of the assay for viral infection which occurred within minutes after viral inoculation. We found that these changes apparently were associated with the viral protein coat rather than with the viral nucleic acid. This seemed to indicate that the viral protein might have some role in the infection process, but further studies will be needed to establish this. During this same period, research at the virus laboratories at the University of California and at the Max Planck Institutes for Biological and Viral Research, Tübingen, Germany, had determined the amino acid sequence of the tobacco mosaic virus protein. The nucleic acid core of one tobacco mosaic virus particle is coated by 2,200 apparently identical protein subunits. Each subunit is composed of 158 amino acids linked together in a long folded chain. While this work revealed the nature of the viral protein, the exact nature of the viral nucleic acid still remained as one of nature's secrets.

IMPROVEMENT IN PLANT VIRAL ASSAY PROCEDURES

1. Use of Scotia instead of Pinto beans for the assay.
2. Use of detached half leaves with incubation between glass plates or on agar surfaces.
3. Pretreatment of bean leaves in 45 C water for 1 minute.
4. Controlled wilting before and after inoculation.
5. Ultrasonic inoculation with carborundum abrasive.

These improvements have also resulted in a significant decrease in previously noted leaf to leaf variation in susceptibility.

THE CELL-FREE SYNTHESIS OF INFECTIOUS TOBACCO MOSAIC VIRAL NUCLEIC ACID BY EXTRACTS FROM INFECTED PLANTS

Background and principles. One of the apparently irrevocable principles of virology was that a virus could multiply only inside a living cell. This concept had become fixed as a part of the definition of a virus in many textbooks. We challenged this view because we believed that it had never been firmly established by adequate experimentation. We believed that the viral RNA that we detected in infected plants was actually the first-formed infective stage of the virus, and we determined to undertake experiments to provide evidence for our concept. The infectious viral RNA can be destroyed by an enzyme called ribonuclease that occurs in all plant and animal cells. We knew that most of the ribonuclease had to be eliminated from plant extracts if we were to demonstrate cell-free synthesis of viral RNA. Research reported by Bonner and others in 1961 indicated that the ribonucleoside-5'-triphosphates were the building blocks that were used by a cell-free system isolated from pea nuclei to make new RNA. Magnesium ions were also apparently essential in this reaction.

We reasoned that if we were to isolate the mechanism that makes a molecule as large as viral RNA, we should take only cellular components

TOBACCO MOSAIC VIRAL NUCLEIC ACID BY EXTRACTS FROM INFECTED PLANTS

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were made comparable with those inside living cells by adding sucrose, calcium chloride, and a pH-buffering chemical called tris (tris hydroxymethyl amino methane).

Early in February 1962, we inoculated a systemically susceptible tobacco plant with tobacco mosaic virus nucleic acid. We used this form of the virus because it is so highly unstable that any inoculum residues remaining on the surfaces of the leaves would lose activity within a few minutes. Thus we insured that no infective inoculum residue would be present to confuse any later analyses of results. Fourteen hours later, the inoculated leaves were harvested. We believed that the use of 14-hour infected leaves would give us the best chance of isolating a highly active viral RNA synthesizing system. As a final safeguard, we added noninfectious viral protein to the container used for the collection of the gel column filtrate. If conditions were suitable, this protein should be available to coat any newly synthesized viral RNA and thus protect it against inactivation by any ribonuclease that might be present.

The infected tobacco leaves were crushed directly over the gel column and the extract washed through the column with our isotonic buffer solution. The drops were collected at the bottom of the column as soon as green-colored chloroplasts appeared in them. The first 10 milliliters collected were reduced to 2.4 milliliters by ultrafiltration through a collodian membrane. The next 25 milliliters collected were centrifuged at $200,000 \times G$ for 2 hours. The resultant pellets were resuspended in the 2.4 milliliters from the ultrafiltration. One milligram of penicillin and 2 milligrams of magnesium chloride were added. After thorough mixing, the extract was divided into two equal lots. One lot, given no further treatment, served as the control. The other lot was given 1 milligram each of the four ribosenucleoside-5'-triphosphates; adenosine triphosphate, guanosine triphosphate, uridine triphosphate, and cytidine triphosphate. The two test tubes were shaken and allowed to stand for 1 hour at room temperature. Then the infectivities in the two tubes were compared by inoculation on opposite halves of 60 glutinosa tobacco leaves. At the points of viral infection this tobacco develops necrotic lesions that can be seen two days later.

Counts revealed that the solution in the control tube produced 77 and thus protect it against inactivation by any ribonuclease that might be present.

The infected tobacco leaves were crushed directly over the gel column and the extract washed through the column with our isotonic buffer solution. The drops were collected at the bottom of the column as soon as green-colored chloroplasts appeared in them. The first 10 milliliters collected were reduced to 2.4 milliliters by ultrafiltration through a collodian membrane. The next 25 milliliters collected were centrifuged at $200,000 \times G$ for 2 hours. The resultant pellets were resuspended in the 2.4 milliliters from the ultrafiltration. One milligram of penicillin and 2 milligrams of magnesium chloride were added. After thorough mixing, the extract was divided into two equal lots. One lot, given no further treatment, served as the control. The other lot was given 1 milligram each of the four ribosenucleoside-5'-triphosphates; adenosine triphosphate, guanosine triphosphate, uridine triphosphate, and cytidine triphosphate. The two test tubes were shaken and allowed to stand for 1 hour at room temperature. Then the infectivities in the two tubes were compared by inoculation on opposite halves of 60 glutinosa tobacco leaves. At the points of viral infection this tobacco develops necrotic lesions that can be seen two days later.

RNA synthesis was studied under varied conditions. The synthesis and subsequent loss of infectious tobacco mosaic viral RNA during a typical one step synthesis experiment are illustrated graphically in figure 1. Figure 2

Virus RNA Synthesis and Loss
In a Cell Free Medium

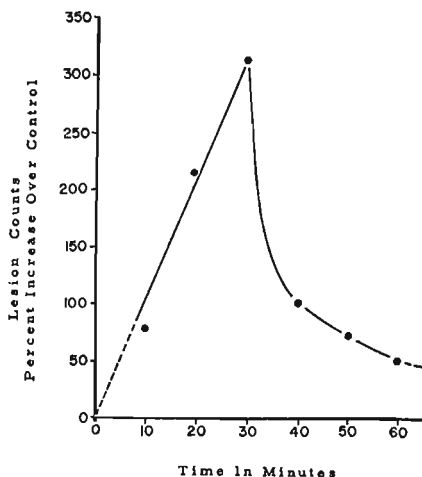


Fig. 1. Synthesis and loss of tobacco mosaic viral RNA during a one-step synthesis experiment using a mixture of tobacco mosaic viral-infected chloroplasts and nuclei with added adenosine, guanosine, uridine, and cytidine triphosphates.

Relation of evolved viral units to repeated incubation of TMV infected tobacco chloroplasts and nuclei in fresh nucleotide substrates.

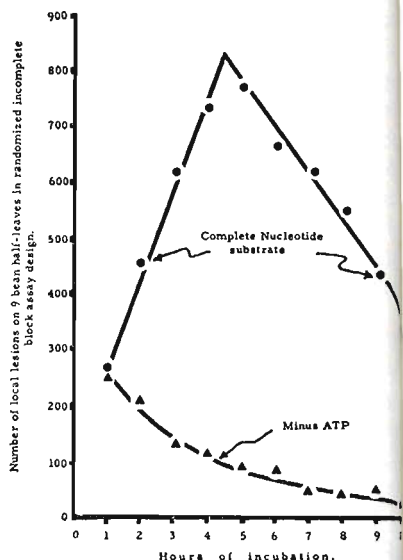


Fig. 2. Synthesis and loss of tobacco mosaic viral RNA during a 10-step repeatedly charged synthesis experiment in which mixtures of infected nuclei and chloroplasts were treated with complete and minus adenosine triphosphate nucleoside supplements.

shows how the repeated addition of complete nucleotide supplements induces a linearly increasing rate of new viral RNA synthesis for 5 hours while a

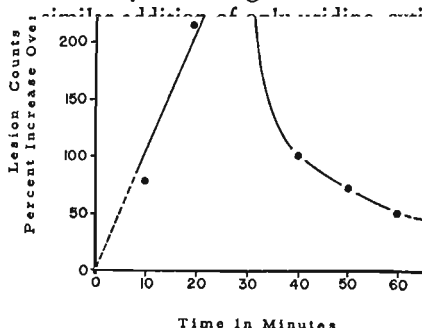


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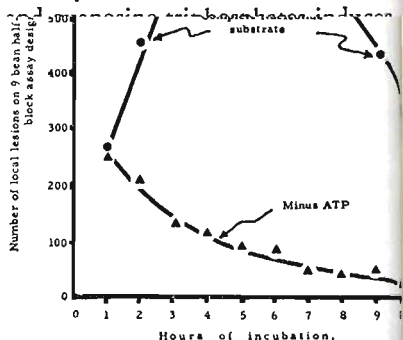


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mosaic viral RNA reported by Kim and Wildman can be explained by the action of their pH 9.5 buffer system in stripping the protein away from intact virus particles to give infectious viral RNA.

Cornuet and Astier in France reported the cell-free synthesis of tobacco mosaic viral RNA in an abstract prepared for the Eighth International Congress of Microbiology (August 20-25, 1962b). They gave no data but reported that the synthesis, which was of relatively weak efficiency, occurred within isolated tobacco mosaic virus infected tobacco nuclei. In November (1962a) they published data which showed that their best preparations made from infected tobacco nuclei gave increases in infectivity varying from 4 to 174 percent with the addition of the same chemical raw materials, the ribose triphosphate nucleosides.

Karasek and Schramm in Germany reported in September 1962 the isolation of a crude enzyme preparation from tobacco mosaic virus-infected tobacco leaves. This, they said, either induced the synthesis of new viral RNA or the completion of partially formed viral RNA in the presence of added ribosenucleoside-5'-triphosphates. Their increased infectivities ranged from 16 to 25 percent.

Our viral RNA-making systems gave significantly greater increases in infectivity than those prepared in the three other laboratories. We attribute this to our unique gel filtration approach which gave us high concentrations of the synthesizing mechanisms after concentration by centrifugation.

Subcellular sites of tobacco mosaic viral RNA synthesis. Instead of pursuing a program of intensive research on the biochemistry of our synthesizing mechanism, we chose to learn more about its intracellular location. We fractionated the material from the gel filtration column by passing it through a series of filters having successively smaller pore sizes. We found that only the residue caught on the filter having the largest pores (5 microns) was capable of making new viral RNA with the additions of the chemical raw materials. These results pointed either to the chloroplasts or the nuclei as the sites of the synthesis. Next we prepared purified preparations of infected nuclei and chloroplasts by density-gradient-centrifuging methods and tested these separately for their synthesizing ability. We found that both a crude enzyme preparation from tobacco mosaic virus-infected tobacco leaves. This, they said, either induced the synthesis of new viral RNA or the completion of partially formed viral RNA in the presence of added ribosenucleoside-5'-triphosphates. Their increased infectivities ranged from 16 to 25 percent.

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It is interesting to note that we have not made any significant improvements in technique since the first experiment. Although we tried modifications we did not find any preparation methods or conditions that gave a more efficient RNA synthesis. The only useful modification has been to replace the ultrafiltration step with a low speed centrifuge concentration procedure.

We did find that it was not necessary to add viral protein to demonstrate RNA synthesis.

Formation of carbon¹⁴-labeled infectious tobacco mosaic viral RNA. Cell-free synthesis can be substantiated by the incorporation of carbon¹⁴-labeled ribosenucleoside-5'-triphosphates into viral RNA which can be coated with viral protein and then purified as whole tobacco mosaic virus. We have now undertaken such synthesis experiments in which carbon¹⁴-labeled adenosine, guanosine, cytidine, and uridine triphosphates have been incorporated. The newly formed RNA was coated with viral protein to form whole virus which was then purified by differential centrifugation techniques. The whole virus was then assayed for infectivity and was measured for radioactive carbon content. Fairly good agreement was obtained between infectivity and radioactivity data. The results of one experiment in which radioactive adenosine was incorporated during 3 consecutive incubations of the same cell-free system are shown in figure 3.

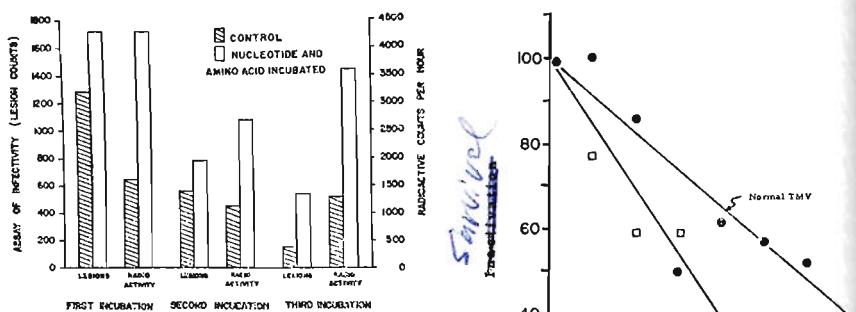


Fig. 3. Repeated biosynthesis of viral RNA by the same synthesizing cell-free

virus which was then purified by differential centrifugation techniques. The whole virus was then assayed for infectivity and was measured for radioactive carbon content. Fairly good agreement was obtained between infectivity and radioactivity data. The results of one experiment in which radioactive adenosine was incorporated during 3 consecutive incubations of the same cell-free system are shown in figure 3.

Formation of 5-fluorouracil-carrying infectious tobacco mosaic viral RNA. We believed that the incorporation of an unusual analog of a purine or pyrimidine base instead of a normal ribosenucleoside-5'-triphosphate could give further proof of the viral RNA synthesis by our cell-free system. From the work of Holoubek (1963) 5-fluorouracil seemed to be the logical choice for our experiments. We assumed that we might have to use the chemical in the nucleoside-5'-triphosphate form if we were to succeed in this substitution.¹ We soon learned that the triphosphate nucleoside form of the chemical was not available and that the special synthesis of a small amount (1/10,000 of a pound) for our experiments might cost \$4,000 or more. We decided instead to determine if our cell-free system could use the freely available 5-fluorouracil form² and we found that it could. The amount of 5-fluorouracil viral RNA formed as detected by infectivity assays was 127 percent of the normal viral RNA formed when complete nucleotide treatments were given to a comparable lot of our cell-free synthesizing system. The lot given the minus uridine triphosphate treatment only synthesized 62 percent of the amount of viral RNA synthesized in the complete nucleotide treatment. Approximately 50 percent substitution of the 5-fluorouracil analog occurred. Noninfectious viral protein was added to the synthesis systems so that whole virus was formed subsequent to viral RNA synthesis. Next we compared the 5-fluorouracil virus with normal tobacco mosaic virus and found that the modified virus was twice as sensitive to ultraviolet inactivation. This difference in inactivation rates is shown in figure 4. By using this analog in place of normal uracil, each substitution has replaced a hydrogen atom with a fluorine atom. Because the atomic weight of fluorine is 19 while that of the replaced hydrogen is only 1, our new viral RNA is heavier than normal tobacco mosaic viral RNA. Therefore we should be able to separate the two types of viral RNA by centrifugation in a cesium chloride density gradient. We are now attempting to do this.

Isolation and partial purification of an enzyme system. In an attempt to isolate the enzyme or enzymes involved in viral RNA formation, we prepared purified tobacco mosaic virus-infected chloroplasts and then ruptured them to release their contents. These were passed through a continuous flow electrophoretic separator to give 48 fractions that were tested. The lot given the minus uridine triphosphate treatment only synthesized 62 percent of the amount of viral RNA synthesized in the complete nucleotide treatment. Approximately 50 percent substitution of the 5-fluorouracil analog occurred. Noninfectious viral protein was added to the synthesis systems so that whole virus was formed subsequent to viral RNA synthesis. Next we compared the 5-fluorouracil virus with normal tobacco mosaic virus and found that the modified virus was twice as sensitive to ultraviolet inactivation. This difference in inactivation rates is shown in figure 4. By using this analog in place of normal uracil, each substitution has replaced a hydrogen atom with a fluorine atom. Because the atomic weight of fluorine is 19 while that of the replaced hydrogen is only 1, our new viral RNA is heavier than normal tobacco mosaic viral RNA. Therefore we should be able to separate the two types of viral RNA by centrifugation in a cesium chloride density gradient. We are now attempting to do this.

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Relations of lesion size, color, and development time to infecting tobacco mosaic viral forms. After observing the thousands of bean leaves used in our viral assays we believed that the appearance of the lesions could be correlated with the type of tobacco mosaic virus inoculum. These differences were noted on the Scotia bean variety. The types of inoculum can apparently be differentiated on heat-treated detached half leaves of Scotia bean incubated on agar gel in glass-covered pans as follows:

Differentiation between whole virus and phenol-prepared viral RNA. With incubation at 28 C most of the phenol-prepared viral RNA lesions were apparent at 16 hours while lesions induced by whole virus were not evident until 18 hours after inoculation. At 32 C this differential increased to 5 hours. At 24 to 30 hours the RNA lesions were noticeably larger. These observations were substantiated by measurements made on thousands of lesions. In one experiment phenol-prepared RNA induced lesions consisting of a series of 4 or 5 concentric necrotic rings while the whole virus inoculum gave only the solidly necrotic lesion types typically associated with tobacco mosaic virus. We are now attempting to determine the conditions which give this type of differentiation. Generally, the RNA lesions appear to have more red color than the whole virus lesions.

Differentiation between phenol-prepared viral RNA and newly synthesized viral RNA. The conditions which induced the formation of the concentric necrotic rings in phenol-prepared viral RNA lesions did not usually induce this characteristic in lesions formed by newly synthesized viral RNA. Instead these lesions resembled those induced by the whole virus. Thus it appears that Scotia bean can differentiate between these two types of viral RNA. The basis for this differentiation is not known. We believe that the newly formed RNA lesions can be differentiated from the whole viral lesions by their earlier appearance. More studies must be made to clarify these relations of lesion character to type of inoculum. We are continuing these studies.

CELL-FREE SYNTHESIS OF INFECTIVE TOBACCO MOSAIC VIRAL NUCLEIC ACID BY EXTRACTS

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CELL-FREE SYNTHESIS OF INFECTIVE TOBACCO MOSAIC VIRAL NUCLEIC ACID BY EXTRACTS

enzymes are produced by the cells only after infection by the viruses. Furthermore these enzymes appear to be specific for each type of virus. The enzyme that makes infectious tobacco mosaic viral RNA cannot make infectious tobacco necrosis viral RNA (Cornuet and Astier 1962b). It then follows that the infecting virus carries a genetic code in its nucleic acid that can direct the synthesis of the viral-RNA-forming enzymes inside cells after infection.

We then reasoned that if we were to succeed in inducing cell-free extracts from normal plants to make new viral RNA, we would first have to induce the formation of viral RNA-forming enzymes which would then form the new viral RNA. Unknown to us, two other laboratories that were working on this problem had tried to synthesize new infectious tobacco mosaic viral RNA with extracts from healthy plants and had failed. Karasek and Schramm (1962) in Germany found that preparations which they made from normal plants completely destroyed added infective viral RNA. Cornuet and Astier (1962b) in France found that 99 percent of the added viral RNA was destroyed by their healthy plant preparations.

Our approach to this phase of the research was on firm ground for we now knew that both infected chloroplasts and nuclei were capable of synthesizing new tobacco mosaic viral RNA. We were determined to isolate the nuclei and chloroplasts from healthy plants for test tube experiments. In a first attempt nuclei were prepared from normal tobacco, inoculated with phenol-prepared tobacco mosaic viral RNA, and incubated with added ribonucleoside-5'-triphosphates. Samples were taken for infectivity assay at hourly intervals for 5 or 6 hours. The results were negative. After viewing the negative results in the greenhouse, we determined to try again, using normal bean nuclei instead of tobacco. We had long ago abandoned the use of tobacco leaves for viral local lesion assays in favor of Pinto bean because the beans could be ready for use within ten days after planting. We knew that the bean leaves reacted to the invading virus by producing necrotic local lesions. Perhaps this necrotic reaction indicated high susceptibility and therefore the normal bean nuclei might succeed in achieving the synthesis of new viral RNA where the tobacco nuclei had apparently failed.

The normal bean nuclei experiment did succeed. Our assays detected and Schramm (1962) in Germany found that preparations which they made from normal plants completely destroyed added infective viral RNA. Cornuet and Astier (1962b) in France found that 99 percent of the added viral RNA was destroyed by their healthy plant preparations.

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SYNTHESIS OF TMV RNA BY NORMAL CHLOROPLASTS

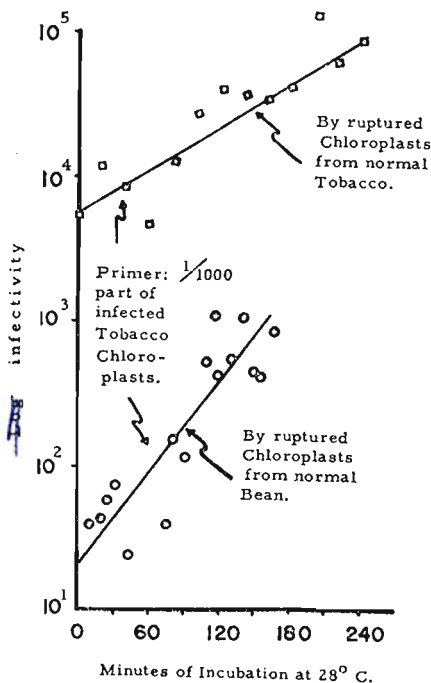


Fig. 5. The synthesis of new infectious tobacco mosaic viral RNA by chloroplasts isolated from normal Pinto bean and normal turkish tobacco primed with small amounts of chloroplasts isolated from tobacco mosaic viral-infected turkish tobacco. All chloroplasts were ruptured by sonic treatment at zero incubation time.

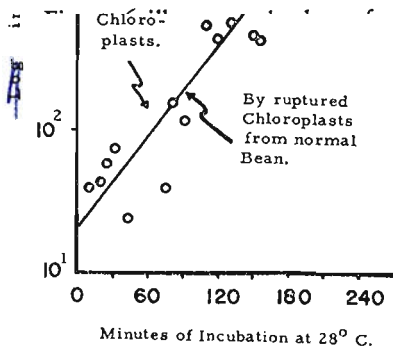


Fig. 5. The synthesis of new infectious tobacco mosaic viral RNA by chloroplasts isolated from normal Pinto bean and normal turkish tobacco primed with small amounts of chloroplasts isolated from tobacco mosaic viral-infected turkish tobacco. All chloroplasts were ruptured by sonic treatment at zero incubation time.

INFECTIVITY OF VARIOUS TMV FOR DURING INCUBATION

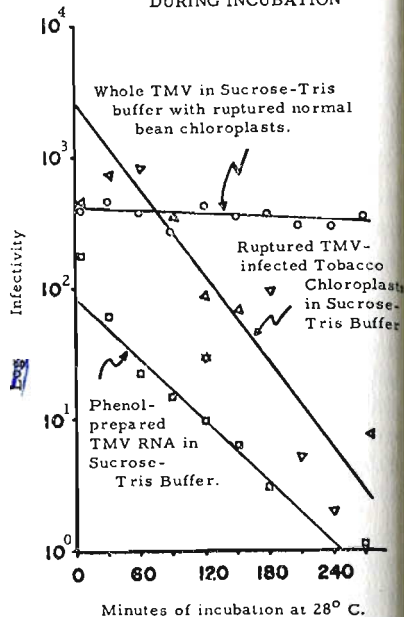


Fig. 6. Effects of incubation of various forms of tobacco mosaic virus in sucrose tris buffer with and without added ruptured normal bean nuclei

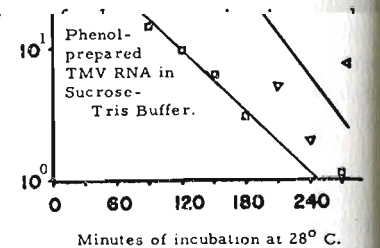


Fig. 6. Effects of incubation of various forms of tobacco mosaic virus in sucrose tris buffer with and without added ruptured normal bean nuclei

results in an inhibition of the synthesis. We were amazed to discover that the cell-free systems prepared from normal plants gave much greater increases in viral RNA than had the infected plant systems. Increases in infectivity of 10- to 1,000-fold were usually obtained. There may be several reasons for this: First, the initial infectivity is nearly impossible to control in the infected systems; however, it can be rigidly controlled in the normal systems because the investigator adds the infectious RNA material to begin the viral RNA-forming reaction. Second, no rate-limiting end products of the synthesis such as pyrophosphate have accumulated in a normal system, but these are probably present in the infected systems. Third, the naturally occurring levels of raw materials in the normal nuclei and chloroplasts may be at near optimum levels while such conditions are probably never obtained in the infected systems with or without additions.

If it is assumed that the mutant form that increased began as one RNA particle, then this mutant must have been increased 500,000,000 fold in the experiment in which it was found. If one particle doubled itself and the 2 gave 4 and 4 gave 8 and so on, by the end of 29 such doublings there would be approximately 500,000,000 copies of the original. Because this occurred in 180 minutes, the average time for the viral-RNA-copying process becomes approximately 6 minutes which is a reasonable figure. We have data from 2 additional experiments that agree with this generation time.

CELL-FREE SYNTHESIS OF WHOLE TOBACCO MOSAIC VIRUS BY EXTRACTS FROM NORMAL PLANTS

THE synthesis of infective tobacco mosaic viral RNA by chloroplasts and nuclei from normal plants after priming with phenol-prepared viral RNA was evidence that the viral RNA primer had induced the formation of a new and specific protein, the viral RNA polymerase enzyme. We reasoned that if the viral RNA primer could do this it could probably also induce the formation of viral coat protein by the same or similar ribosomes

If it is assumed that the mutant form that increased began as one RNA particle, then this mutant must have been increased 500,000,000 fold in the experiment in which it was found. If one particle doubled itself and the 2 gave 4 and 4 gave 8 and so on, by the end of 29 such doublings there would be approximately 500,000,000 copies of the original. Because this occurred in 180 minutes, the average time for the viral-RNA-copying process becomes approximately 6 minutes which is a reasonable figure. We have data from 2 additional experiments that agree with this generation time.

CELL-FREE SYNTHESIS OF WHOLE TOBACCO MOSAIC VIRUS BY EXTRACTS FROM NORMAL PLANTS

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28 C and stirred continuously for 1 week. One week after the last addition the chloroplast and nuclear slurry was removed and a small portion was assayed on 12 bean half leaves. This assay induced the formation of 30 lesions.

This experiment was repeated with the normal bean nuclei and chloroplasts being added continuously for 24 hours. After this the mixture was again stirred for 1 week before being assayed for virus. During the stirring period a malfunction of the constant temperature regulator increased the temperature of the mixture to 55 C for an undetermined period of time. A small portion of the incubated mixture was again assayed on 12 half leaves and induced the formation of 43 lesions.

In a third experiment a mixture of normal bean chloroplasts and nuclei was suspended in our standard buffer system and mixed with 250 micrograms of phenol-prepared RNA, 4 ribosenucleoside-5'-triphosphates, and 19 amino acids. The mixture was placed in a small erlenmeyer flask and held at room temperature with frequent manual stirring. Each day for 1 week additional normal bean chloroplasts, nuclei, amino acids, and nucleoside-5'-triphosphates were added. On the ninth day a small portion of the slurry was assayed by inoculation on 12 bean half leaves. This sample induced the formation of 5,508 lesions.

This third experiment differed from the first two chiefly in that amino acids were added. We believe that the significant increase (over the first two experiments) in the amount of infective virus formed can probably be attributed to the action of the added amino acids. These were available for the synthesis of much greater amounts of viral coat protein.

An application of the bentonite flocculation serological test of Scott and others (1963) provided rather conclusive evidence that our cell-free system was synthesizing tobacco mosaic viral coat protein. The incubated mixture from the third experiment gave a strong flocculation of a tobacco mosaic virus antibody bentonite preparation as shown in table 1. This mixture gave no reaction to the test at zero incubation time.

TABLE 1. Serological reaction of a cell-free tobacco mosaic virus-synthesizing system consisting of normal bean chloroplasts and nuclei suspended in isotonic sucrose-tris buffer, (pH 7.2), primed with phenol-prepared viral RNA and incubated with periodically added ribosenucleoside-5'-triphosphates, amino acids, and normal bean nuclei and chloroplasts. Diluted samples were mixed with a tobacco mosaic virus antibody bentonite preparation and observed after 1 hour of shaking.

Time of testing	Dilution factor					
	5	25	125	625	3,125	15,625
Zero incubation	—	—	—	—	—	—
12 days' incubation at room temperature	?	+++++	+++	++	+	—

— No reaction
+ Positive reaction

To obtain further proof of whole virus synthesis, the virus was isolated from the chloroplast-nuclear mixture using the method of Venekamp (paper in preparation for publication), atomized on Formvar-covered electron microscope grids, and photographed with the electron microscope. The newly synthesized viral particles are shown on the cover.

In the third experiment 1.7 milligrams of purified whole virus were recovered. In a fourth experiment primed with 300 micrograms of viral RNA, carbon¹⁴-labeled 5-fluorouracil was substituted for the normal uridine-5'-triphosphate. After 72 hours of incubation 5.3 milligrams of purified whole virus were isolated. In a fifth experiment primed with 1 microgram of viral RNA, carbon¹⁴-labeled uridine triphosphate was substituted for normal uridine-5'-triphosphate. After 72 hours of incubation 5.4 milligrams of purified whole virus were isolated. Carbon¹⁴ radiation recorded at levels of 50 and 459 counts per milligram of whole virus per minute indicated that the incorporation of 5-fluorouracil and uridine-5' triphosphate, respectively, had occurred during the cell-free synthesis reactions. These experiments prove that the whole infectious tobacco mosaic viral unit can be synthesized by our cell-free system. The synthesis of the RNA which had been demonstrated repeatedly in our earlier work was again verified by the incorporation of carbon¹⁴-labeled 5-fluorouracil and uridine triphosphate and by the fact that experiment 5, which was primed with 1 microgram of viral RNA, yielded 270 micrograms of viral RNA in the isolated purified whole virus. The actual increases were much greater because our virus isolation and purification procedures did not recover all of the virus from the cell-free systems. The occurrence of the virus protein was proved by the strong agglutination with viral antiserum and by the fact that highly stable infective viral rods were formed. All of our serological tests of normal chloroplasts and nuclei and viral RNA have been negative. The presence of the viral protein then is evidence of its synthesis by the system and the presence of the viral rods demonstrated by electron microscopy is evidence that the viral RNA and protein were assembled to form whole viral units.

BIOCHEMISTRY OF THE TOBACCO MOSAIC VIRUS FORMATION PROCESS

WE ARE now able to describe the several processes involved in virus formation and to partially express these in terms of chemical equations. For the simplicity of discussion these processes are treated separately although it is highly probable that all of these reactions are proceeding simultaneously.

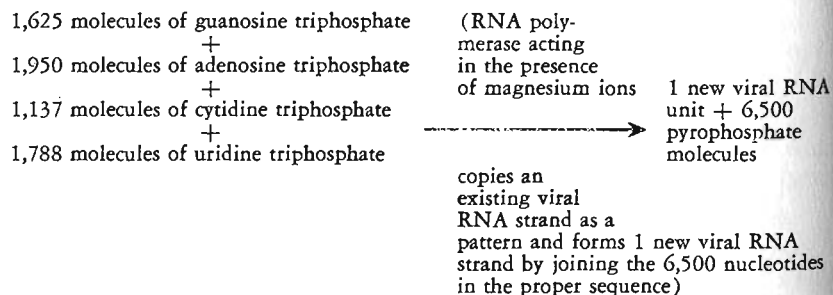
Formation of viral RNA polymerase. A viral ribonucleic acid chain diffuses inside either a chloroplast or a nucleus. The portion of the nucleic acid carrying the genetic code which directs the synthesis of the viral RNA

polymerase enzyme comes in contact with the surface of a ribosome, a tiny particle containing ribonucleic acid and protein. The new polymerase enzyme is built according to the coded directions of the viral RNA. This enzyme protein is assembled within seconds by protein enzyme systems and soluble RNA molecules which attach to the required amino acids to move them into the proper position for building the new enzyme protein. When completed, polymerase enzyme is released, and the viral RNA ribosome site may be available for the formation of a second enzyme unit in the same manner. Or perhaps it builds a unit of viral coat protein by continuing to read another portion of the viral RNA coded message.

Formation of viral ribonucleic acid. The polymerase enzyme probably moves by diffusion within the chloroplast or nucleus until it contacts a free tobacco mosaic viral RNA chain. It probably attaches to one end of the viral RNA chain because its chemical structure allows it to align itself closely with the RNA molecule. This ability for close alignment could explain the high specificity of the viral RNA - polymerase relation. In some now unknown manner the enzyme builds a new viral RNA chain by adding the nucleotides one at a time in the proper sequence. As it does this, the proper nucleotide to be added next falls into position and is added to the growing RNA chain. The geometry of the complex of enzyme, original RNA pattern, and newly forming RNA probably permits only the proper nucleotide to move into the assembly position at the proper time. The raw materials are the four ribonucleoside-5'-triphosphates: adenosine triphosphate, guanosine triphosphate, cytidine triphosphate, and uridine triphosphate. The energy for the coupling reaction is already present in the triphosphate bonds. The addition of each nucleotide releases one unit of pyrophosphate.

Stanley and Valens (1961) have stated that one tobacco mosaic virus RNA particle consists of approximately 6,500 purine and pyrimidine bases. Holoubek (1963) gives the following ratios for the bases in tobacco mosaic virus: guanosine, 1.00; adenine, 1.21; cytosine 0.73; uracil 1.10. This information can be used to calculate the approximate numbers of each type of nucleotide that would be needed for the synthesis of one viral RNA unit.

The reaction by which one viral RNA unit is assembled might then be written in the following manner:



The exact sequence of the 6,500 bases in the RNA chain is now unknown, but techniques for determining the sequence are being developed. At the end of the copying process both strands of the RNA probably become available again for a repetition of the copying process by the polymerase enzyme systems. This could explain the phenomenal rate of growth that we have observed in our test tubes. One unit is copied to give 2 then the 2 can be copied to give 4 and the 4 to give 8 and so on. In 30 cycles of copying, 1 particle can be increased to more than 1 billion. We have observed that on the average the formation of the 6,500 chemical unions to make one viral RNA strand requires about 6 minutes. If it were $6\frac{1}{2}$ minutes, this would be 1,000 per minute or about 17 per second. Each time a nucleotide unit is added, the proper one must be chosen from four available types. That the polymerase enzyme makes this proper choice with the molecules being chemically joined to the growing RNA chain at the rate of 17 per second reveals a truly amazing phenomenon. When a viral RNA strand becomes coated with the viral protein, it is no longer available to serve as a pattern for the synthesis of new viral RNA or as a code for the formation of polymerase enzyme or viral protein. It is in a dormant, resistant, resting condition and can withstand all sorts of adversities until by chance it is placed in a susceptible cell where it can again initiate its usual reproductive cycle after the removal of its protein coat.

Formation of viral coat protein. A viral ribonucleic acid strand also carries a genetic code which can direct the synthesis of the viral coat protein. The portion of the RNA strand carrying this code comes in contact with the surface of a ribosome inside a chloroplast or a nucleus in our cell-free system. Viral coat protein is built according to the coded directions of the viral RNA and assembled within seconds by the same protein-assembling enzyme systems, soluble RNA molecules, and amino acids that have already been described for the polymerase formation process. However coat protein is built instead of viral polymerase because this particular portion of the viral genetic code calls for coat protein. Stanley and Valens (1961) list the amino acids needed to make one unit of coat protein giving the exact sequence of each.

The reaction for the synthesis of one unit of viral coat protein might be written as follows:

		18 molecules of aspartic acid	
		+	
		16 molecules of glutamic acid	
		+	
		16 molecules of serine	
		+	
		16 molecules of threonine	
		+	
		14 molecules of alanine	
		+	
		14 molecules of valine	
	+	+	
158 molecules of		12 molecules of leucine	
adenosine triphosphate		+	
		11 molecules of arginine	
		+	
		9 molecules of isoleucine	
		+	
		8 molecules of proline	
		+	
		8 molecules of phenylalanine	
		+	
		6 molecules of glycine	
		+	
		4 molecules of tyrosine	
		+	
		3 molecules of tryptophan	
		+	
		2 molecules of lysine	
		+	
		1 molecule of cysteine	

158 specific activating enzymes	$\xrightarrow[\text{ions}]{\text{manganese}}$	158 amino acid- adenylate- enzyme complexes	+	158 molecules of pyrophosphate	(1)
---------------------------------------	---	--	---	---	-----

158 amino acid-adenylate- enzyme complexes	+	158 specific soluble RNAs	\longrightarrow	158 amino acid-soluble RNAs	+	
158 molecules of adenosine	+	158 specific activation enzymes				(2)
monophosphate						

158 amino acid-
soluble
RNAs

At the viral RNA-ribosome juncture,
in the presence of adenosine
triphosphate, guanosine triphosphate,
magnesium ions, and reduced glutathion

1 unit of viral
coat protein +
157 molecules of
water (3)
+ 158
soluble
RNAs

Assembly of whole virus. The viral RNA appears to attract the viral coat protein at suitable ionic strengths and at pH values below 7. The protein units also have an attraction for each other under these conditions. They are held to each other by relatively weak bonds; as they aggregate they lose water that is bound to their contact surfaces (Lauffer et al. 1961). Twenty two hundred of these subunits coat the viral RNA in a spiraling manner to form a helical structure that has the appearance of a rigid rod 17 times as long as it is thick.

CELL-FREE SYNTHESIS OF INFECTIOUS RNA OF CUCUMBER MOSAIC VIRUS

After several months of research with the tobacco mosaic viral RNA-synthesizing systems, we began to try similar approaches to demonstrate the cell-free synthesis of cucumber mosaic virus.³ Because cucumber mosaic virus is quite unstable and easily inactivated, the demonstration of the cell-free synthesis of its RNA proved difficult. The gel filtration techniques that worked so well for tobacco mosaic virus did not yield results with cucumber mosaic virus. Eventually it was discovered that infected chloroplasts prepared by density gradient centrifugation procedures were capable of synthesizing new infective cucumber mosaic viral RNA, but infected nuclei were not. This contrasted with the tobacco mosaic virus results where both the nuclei and chloroplasts were involved in the synthesis.

CELL-FREE SYNTHESIS OF INFECTIOUS RNA OF WESTERN EQUINE ENCEPHALITIS VIRUS

BEGINNING in August of 1962, Dr. J. Storz of the Veterinary Science Department cooperated in experiments to demonstrate the cell-free biosynthesis of an infectious animal viral RNA. We decided that western equine encephalitis might offer the best possible chance for success because effective assay methods had already been devised for the RNA of this virus.^{4 5}

The animal systems used were obtained from developing chicken embryos. Embryos were infected with the virus 3 to 8 hours before being harvested. We used the same type of particulate gel columns for the preparation of cell-free nuclei, mitochondria, and other subcellular elements from crushed normal and infected chicken embryos. The infected subcellular ele-

³ This became the thesis for Men Hui Lee.

⁴ We obtained a culture of the virus from D. W. Hill at the University of Utah.

⁵ We were assisted chiefly by Antonina Mikulska-Macheta in these experiments.

ments were either incubated alone, with or without the added four ribonucleoside-5'-triphosphates, or were mixed with similarly prepared normal subcellular fractions. One of our major problems was that we did not anticipate the initial infectivity and subsequent amount of viral RNA synthesis that occurred in our test tubes and therefore did not dilute the assay samples enough during the course of the earliest experiments. Figure 7 shows the titers of infectivity found in various incubated preparations in selected animal virus experiments. In these experiments the mixture of infected and normal components usually resulted in 10,000- to 100,000-fold increases in infective viral RNA during about 2 hours of incubation. Because such rapid increases had never been shown for this virus using whole-cell culture techniques, the facts seemed to indicate a truly different synthesis. By the end of 1962 we had completed at least four experiments with excellent evidence of marked viral RNA synthesis. The findings were reported briefly in a paper presented at the symposium on "Viruses, Nucleic Acids, and Cancer" (Cochran 1963) held in Houston, Texas, February 21, 1963. Following the presentation, Huppert from France reported on experiments in his laboratory in which the infectious RNA of encephalomyocarditis virus was apparently synthesized by cell-free preparations during test tube incubation to give a 3,000 fold increase in infectivity. A more complete account of these experiments was later published in France. We presented a more detailed account of our western equine encephalitis RNA synthesis experiments at the Federation meetings in Atlantic City in April and also submitted an account to *Nature* (Cochran et al. 1963b) for possible publication. Glasky and Holper (1963) claimed to have demonstrated the synthesis of infectious material using cell-free systems prepared from calf kidney cells infected with either influenza or Newcastle disease viruses. They cited the production of hemagglutinating ability in their preparations as evidence of infectiousness. Since hemagglutination is a property of the outer surface of the viral protein coat and infectivity is an inherent property of the viral nucleic acid, the two properties, while frequently associated, are not synonymous. The hemagglutinating effect cannot be claimed as evidence for the production of infectivity.

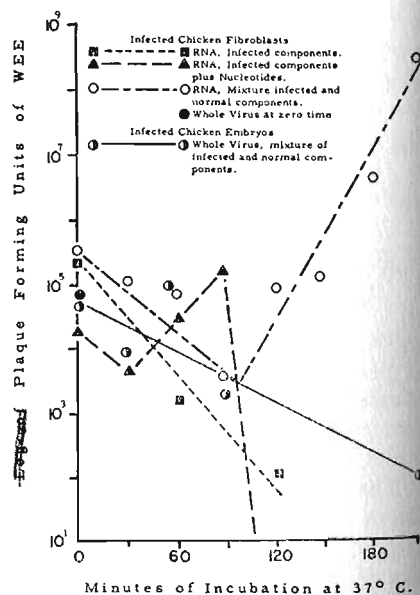


Fig. 7. Titers of western equine encephalitis virus and viral RNA during incubation of cell-free subcellular components from infected chicken embryos or fibroblasts and normal chicken embryos.

SUMMARY OF EVIDENCE FOR CELL-FREE SYNTHESIS OF VIRAL RNA AND WHOLE VIRUS

1. Infected chloroplasts or nuclei, separated or mixed, ruptured or intact, synthesized new infective tobacco mosaic viral RNA giving as much as 3- to 4-fold increases in infectivity during 30 minutes of incubation with added ribosenucleoside-5'-triphosphates.
2. The rate of new cell-free tobacco mosaic viral RNA synthesis increases linearly for 5 hours during a repeated charging of the synthesizing system with fresh nucleotides at hourly intervals.
3. The withholding of any one of the four ribosenucleoside-5'-triphosphates inhibits the tobacco mosaic viral RNA synthesis by the cell-free system.
4. The cell-free system synthesizes new radioactive tobacco mosaic viral RNA if any of the four ribosenucleoside-5'-triphosphates is labeled with C¹⁴.
5. The cell-free system synthesizes new infective 5-fluorouracil tobacco mosaic viral RNA if 5-fluorouracil is substituted for uridine triphosphate. The newly synthesized 5-fluorouracil viral RNA is twice as sensitive as normal tobacco mosaic viral RNA to ultraviolet inactivation and should also separate from the normal during density gradient centrifugation because of the substitutions of heavier fluorine atoms for hydrogen in the RNA molecule.
6. Preparations of normal bean or tobacco nuclei or chloroplasts, ruptured or intact, mixed or separated, synthesized new infective tobacco mosaic viral RNA after priming with phenol-prepared viral RNA or viral RNA from infected chloroplasts or nuclei. The increase in infectivity was sometimes 1,000 fold or greater.
7. In a tobacco mosaic viral RNA synthesis experiment using nuclei from normal Pinto bean a new mutant was synthesized along with the common viral RNA. If the mutant started as one particle, then the increase was 500,000,000-fold giving a calculated average generation time of 6 minutes.
8. The newly synthesized tobacco mosaic viral RNA from our cell-free systems appears to give characteristically smaller lesions on bean than RNA prepared from whole virus by the phenol method. Lesions from both types of RNA appeared about 2 hours sooner than lesions induced in Scotia bean by whole virus.
9. The cell-free synthesis of relatively large amounts of new tobacco mosaic viral RNA by normal chloroplasts and nuclei provides evidence that the same cell-free system synthesized significant amounts of a new specific protein. This protein is the viral RNA polymerase system which was probably formed before and concurrently with the formation of the new infective viral RNA.

10. We have isolated and partially purified this tobacco mosaic viral RNA polymerase enzyme system from tobacco mosaic virus-infected tobacco chloroplasts. It will make new infective viral RNA units from the four ribonucleoside-5'-triphosphates in a test tube when a small amount of viral RNA is added to act as a master pattern for the synthesis.
11. A cell-free system of normal bean nuclei and chloroplasts mixed with phenol-prepared tobacco mosaic viral RNA primer, ribonucleoside-5'-triphosphates and amino acids was slowly fed with additional normal chloroplasts, nuclei, triphosphate nucleosides, and amino acids. This system synthesized whole tobacco mosaic virus which retained its infectivity while being stirred within the chloroplast and nuclear mixture at a temperature of 28 C or higher for 7 days. Tobacco mosaic virus rod-shaped particles were found in these systems.
12. Cell-free preparations of chloroplasts from cucumber mosaic-infected cowpea plants synthesized new infective cucumber mosaic viral RNA from added ribonucleoside-5'-triphosphates.
13. Mixtures of cell-free preparations of nuclei, mitochondria, and subcellular elements including endoplasmic reticulum from western-equine-encephalomyelitis virus-infected and normal chicken embryos synthesized new infectious RNA of this virus with as much as a 100,000-fold increase in infectivity during 2 hours of test-tube incubation.

WHAT WILL THE FUTURE BRING?

1. In the future the detailed chemistry of the virus formation processes will be thoroughly understood.
2. Using this new knowledge about the chemistry of the virus formation processes, new and practical chemical methods of controlling virus diseases will be devised.
3. Because many cancers are, possibly, caused by viruses, effective chemical viral controls may lead to cancer prevention.
4. Facts obtained from studies of viral nucleic acid synthesis reactions may lead directly to a practical control for cancer by revealing new points of vulnerability in the nucleic acid formation processes in cancer cells.
5. The sequence of the bases in RNA and DNA will be worked out so that eventually base sequence can be related to heritable characteristics in viruses and other organisms.
6. This information can lead to the determination of the cause of genetic defects in organisms including man, and treatment methods can be devised to overcome these defects.
7. Eventually, through chemistry, hair and eye color and other characteristics of offspring will be predetermined or manipulated.

8. Biochemists will learn how to link nucleotides together in the desired sequence to produce the equivalent of new viral RNA or DNA molecules and other entirely new forms of life.

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TWENTY-EIGHTH HONOR LECTURE DELIVERED AT THE UNIVERSITY

November 26, 1963

A BASIC objective of the Faculty Association of the Utah State University, in the words of its constitution, is

To encourage intellectual growth and development of its members by sponsoring and arranging for the publication of two annual faculty lectures in the fields of (a) the biological and exact sciences, including engineering, called the Annual Faculty Honor Lecture in the Natural Sciences, and (b) the humanities and social sciences, including education and business administration, called the Annual Faculty Honor Lecture in the Humanities.

The administration of the University is sympathetic with these aims and shares the cost of publishing and distributing these lectures.

Lecturers are chosen by a standing committee of the Faculty Association. Among the factors considered by the committee in choosing lecturers are, in the words of the constitution:

(1) creative activity in the field of the proposed lecture; (2) publication of research through recognized channels in the fields of the proposed lecture; (3) outstanding teaching over an extended period of years; (4) personal influence in developing the characters of students.

Dr. Cochran was selected by the committee to deliver the Faculty Honor Lecture in the Natural Sciences. On behalf of the members of the Association we are happy to present this paper: THE SECRETS OF VIRAL REPRODUCTION.

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